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14. ABSTRACT

Report developed under STTR contract for topic "ARMY01-T005". The purpose of the project was to demonstrate feasibility of developing transgenic chickens by methods invented by the Contractor and RI. The method involved construction of a patented transposon-based genetic vector and injecting this into the testes of young roosters. A total of 88 white leghorn roosters underwent surgery and had the vector injected (~5 ug DNA) into their testes at 4-14 weeks of age. Upon attainment of sexual maturity at ~20 weeks, a spermatozoa sample was obtained and PCR analysis performed to ascertain incorporation of the gene. Confirmed 'transgenic' spermatozoa were detected in eight mature roosters (~9%) which demonstrates feasibility of the method. These 'transgenic' roosters could be expected, by simple breeding, to generate female offspring also carrying the gene. This makes possible the prospect of 'gene pharming' from the white of eggs from hens carrying a transgene coding for a variety of pharmaceuticals or peptides.

15. SUBJECT TERMS

STTR Report---- transgenic---- chickens---genetic biomanufacturing---transposon---testes

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TransWestTech, Inc. Final report
Phase I STTR Contract # DAAD19-02-C-0016

The principal objective of Phase I was to (a) develop a construct based on the Co-investigator's transposon vector for use in poultry, and (b) to develop a method for injecting this construct into the testes of immature male chickens. These males would have transposon DNA incorporated into primary spermatagonia to generate sperm capable producing transgenic offspring. Some of these offspring would be hens with the ability to express proteins of this construct into the whites of their eggs.

We have accomplished what was proposed regarding gene delivery through intra-testicular injection. Using chicken husbandry and surgical methods developed during Phase I, we have successfully injected the first gene construct into the testes of 84 immature and 4 mature White Leghorn roosters, plus 4 controls. The first successfully constructed vector was designated pBTnMCS(CMV/Red) and consists of the following elements (Figure 1).

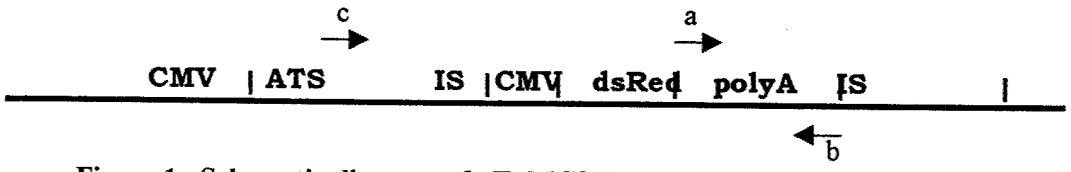

Figure 1. Schematic diagram of pTnMCS/dsRed showing the key elements of the vector. Primer set "a" and "b" amplify a 500 bp fragment of dsRed. Primer set "c" and "b" determine transgene incorporation. CMV = cytomegalovirus promoter; ATS = transposase; Op = ovalbumin promoter; and IS = insertion sequences.

A cytomegalovirus immediate early promoter (CMV IE) controlled expression of both the transposase gene and the dsRed gene on the plasmid. The dsRed gene was chosen to allow for rapid identification cells carrying the transgene by detection of red fluorescence. Any bird with red fluorescent cells would be tested using PCR to confirm transgene incorporation.

A specific PCR protocol was developed in the Co-investigator's laboratory based on the primer pair (primer set "a" and "b", Figure 1) that amplified a unique 500bp band within the dsRed gene, which contained no possible confounding chicken sequences. In addition, PCR controls were routinely performed using primers (proprietary) unique to the transposon elements of the designed construct (primer set "b" and "c", Figure 1). Because of the manner by which the vector was designed, if the dsRed gene became incorporated into the sperm, the PCR would be negative, thus demonstrating transposition of the gene into the chicken chromosome. Other negative (control rooster sperm) and positive controls (plasmid DNA) were simultaneously performed with each group of samples analyzed. During each PCR, these positive and negative controls gave the correct response, indicating that the PCR procedures were working properly.

For DNA injections, experimental males (white leghorns) were obtained from commercial sources at one day of age and reared in brooders or floor pens using good animal husbandry practices. They were given water and standard Starter Chick chow ad lib and kept initially in a 23:1 hour light/dark cycle, stepping down at weekly intervals to a 15:8 hour light/dark cycle. All experimental and husbandry procedures have been approved by the institution's IACUC.

At the appropriate age, individual males had their food removed for 12-18 hours. The males were anesthetized with isofluorane using a 100 mL plastic device placed over their face which contained cotton saturated with 2 mL isofluorane, which was repeated at intervals needed to maintain surgical anesthesia. Birds were positioned in lateral recumbency and restrained with braided parachute cord, applying gentle traction to the wings and feet in opposite directions. This allows for a simple lateral approach through the abdomen to allow access to the testes area. The

abdominal area was prepared with 0.5% chlorohexidine surgical scrub and alcohol and a 3 cm skin incision made between the last two ribs exposing intercostal musculature. The procedure continued via blunt dissection through the peritoneum into the abdomen. A small animal retractor was used to spread the ribs to expose and visualize the testis. This process takes 5-15 minutes and is a similar approach to the procedure for caponization.

Immediately prior to injection into the birds, the appropriate amount of DNA was mixed with Superfect (1:3 ratio). The DNA solution was injected into each testis in 3 sites. The injection device used consists of a 250 μL syringe and a 25 gauge hypodermic needle bent to a 80 degree angle, as this has been found necessary for accurate injection. Approximately 150-200 μL of a carrier solution containing 2-5 μg of the test gene construct (pTnMCS(CMV/Red) were injected with 3 equal volumes into 3 separate locations of each testes. A total of 88 birds were included in the study, Injections were made at 4 weeks (n=10), 6 weeks (n=14), 8 weeks (n=16), 10 weeks (n=17), 12 (n=5) weeks, 14 weeks (n=22) and 52+ weeks (adults, n=4). Four control birds (saline and Super-Fect only) were maintained during the period. Fourteen birds died or were killed prior to sampling.

Young birds received approximately 2.5 μg DNA per testis while the older birds received 5 μg. We estimate that our procedure resulted in about 10^4 -10^5 DNA molecules per spermatogonium being injected into each testicle.

At 18 to 30 weeks of age, sperm samples from each bird were evaluated for potential transgene insertion, (successful testis transfection), by PCR of sperm. Chickens were housed in individual cages at least 3 days prior to collection of sperm samples. The sperm was obtained from each rooster by manual manipulation. (Burrows & Quinn 1937) Semen was collected using a disposable polyethylene transfer pipette (SAMCO Scientific Corp, San Fernando, CA Cat #691) and mixed with 500 μL PBS and stored at 4°C prior to evaluation.

Fresh sperm and testes samples were forwarded to the Co-investigator's laboratory at Louisiana State University for PCR analysis. A PCR procedure was developed to test for the incorporation of the test gene, a chromophore, called dsRed. The PCR protocol was based on the primer pair, which amplified a unique 500bp band within the dsRed gene for the reasons noted above. In addition, PCR controls were routinely run using primers unique to the transposition elements.

For PCR evaluation, the sperm DNA was extracted using a Qiagen Blood Tissue Kit (Qiagen, Inc., Chatsworth, CA) and aliquots frozen for future reference. The PCR protocol, utilizing the appropriate primers, is described above. Samples were obtained from roosters beginning about 18 weeks of age. Finding a PCR positive sperm sample after 2 months or more post-injection would constitute strong evidence of integration. The percentage of sperm which have been transformed is expected to be in the range of 2 –10 percent. Breeding experiments will be required to determine if this has been achieved.

After the third sample, each bird was killed and a sperm sample and testes were taken for PCR.

Results

The reporter gene was successfully produced early in Phase I by the Co-investigator and was supplied to TWT in Bozeman for injection into the chicks. The construct was injected successfully into each testis of 88 birds, including four adult birds as an added group for comparison to the immature chicks, as shown below:

Table 1. Summary of roosters injected with test DNA vector

Age at injection (Weeks)	Number of birds injected
4	10
6	14
8	16
10	17
12	5
14	22
52+ (Adult)	4
Control	4

We lost four birds either during surgery or within one day after, from bleeding in one case and strangulated intestine in another. Ten others died or were sacrificed because of injury.

Seventeen PCR-positive samples were obtained after approximately 2-3 months post-transfection. These, however, were apparently transient, since repeat tests on these birds showed no PCR positive result. Subsequently, however, eight roosters, six of which had been injected at age 14 weeks, have had confirmed PCR positive spermatozoa 4 months post-transfection. Thus, these constitute strong direct evidence that the transgene was incorporated into the genome of at least a portion of the bird's spermatagonia and should be transmitted by breeding. Breeding of the positive birds has been initiated. The unequivocal location of the gene within the target DNA coupled with the excision of the transposon elements, as designed, match the criteria demanded for transgenic insertion. Any PCR positive for the vector observed in this analysis, would have indicated that residual injected (non-integrated) construct was present and the transgene had not incorporated. The Co-investigator has obtained preliminary evidence that the dsRed gene product expressed at high levels is toxic to the recipient cells and therefore providing less evidence for transgenesis than expected. In spite of not having the convenience of this visual confirmation, the PCR evidence obtained is sufficient to indicate that the Phase I goal has been met. A Provisional Patent on the method has been written and will be submitted.

Recently, the Co-investigator has completed a transposon vector in which the transposase was optimized for avian expression. Preliminary use of this vector in Japanese quail as a model species has resulted in 90% of the birds carrying the transgene incorporated in their genome. Breeding studies are underway to determine the transmission rate to offspring and the first experiment with the new vector in chickens is in progress. *These results will allow immediate application of this technology to develop constructs for Phase II.*

Thus we have demonstrated the possibility of routinely and efficiently generating transgenic avians with the possibility of creating transgenic hens that secrete valuable polypeptides into their egg white. The objective of Phase II is to demonstrate the feasibility of producing a commercially valuable protein-based pharmaceutical in egg white with our technology. For this next phase, we will simply modify the basic vector to carry a gene for a commercial protein (driven by the ovalbumin promoter) to direct expression of that protein in the oviduct of the F1 transgenic hens.